



PATENT  
Atty. Docket No. 2599

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#25

APPLICANT : Russell G. Higuchi  
SERIAL NO. : 07/695,201 GROUP ART UNIT: 1814  
FILED : May 2, 1991 EXAMINER: R. Prouty  
DOCKET NO. : 2599  
TITLE : HOMOGENEOUS METHODS FOR NUCLEIC ACID  
AMPLIFICATION AND DETECTION

Signature

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Hon. Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

I, Dr. Richard D. Abramson, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. §1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

2. I received a Ph.D. in Biochemistry from Case Western Reserve University in 1987. A copy of my *curriculum vitae* is attached as Exhibit 1 and is incorporated herein by reference.

3. I am presently employed by Roche Molecular Systems as a Research Investigator in the Program in Core Research. I am primarily responsible for studying the enzymological properties of thermostable DNA polymerases and their use in DNA sequencing and polymerase chain reaction (PCR) technologies.

4. I have read and am familiar with the contents of the subject patent application. I understand that the Examiner has a single basis for rejecting the pending claims. The claims are rejected as obvious in view of a combination of three references. Mullis *et al.* are relied upon for teaching PCR. Sutherland *et al.* are relied upon for disclosing the use of fluorescent dyes such as ethidium bromide (EtBr) for measuring the polymerization activity of DNA polymerases. Kaledin *et al.* are relied upon to show that the minimum concentration of EtBr which "inhibits *Thermus flavus* DNA polymerase is 5  $\mu$ M and that half maximal inhibition requires 23  $\mu$ M ethidium bromide."

5. In the previous Response, Applicant provided Kaledin to explain why one of skill would not add EtBr to a PCR mixture due to the inhibitory effects EtBr can have on 3' extension. The Examiner then relied on Kaledin to assert that its teachings would motivate one of skill to use low concentrations of EtBr to avoid inhibition of the Mullis PCR. Applicant will explain that the Examiner's interpretation of Kaledin *et al.* is based on a misapprehension and that the conclusion regarding obviousness is based upon an oversimplification of the underlying science.

6. There are several scientifically valid reasons why one of skill would not view Kaledin as suggesting the use of low concentrations of EtBr in a Mullis PCR mixture, based on a correct understanding of the mechanism of EtBr inhibition of DNA polymerization. The Examiner's interpretation of Kaledin *et al.* is based on a misapprehension. The polymerase molecule *per se* is not inhibited by EtBr. It is the *extension of the primer-template complex* that is inhibited by EtBr binding to double stranded regions of the DNA substrate, and it is the EtBr:DNA ratio that determines if the polymerase assay will be inhibited, not the absolute amount of EtBr in the reaction or the ratio of EtBr to enzyme.

First, I have compared the relative ratios of EtBr:DNA in the Kaledin reference to the various examples disclosed in the subject specification. My analysis reveals that the claimed invention operates, without inhibition, at ratios reported by Kaledin to be 50% inhibitory of a polymerase reaction. Furthermore, at ratios equal to or below that reported by Kaledin to be minimally inhibited, fluorescence of the PCR amplification falls below the minimal level of sensitivity which is easily and readily detectable, thus limiting the usefulness of the invention. These surprising results are direct evidence of the non-obviousness of this invention.

Secondly, a closer examination of the components of the PCR reveals that the majority of the nucleic acid is single stranded primer, in contrast to the Kaledin assays, where the

majority of the nucleic acid is nicked and gapped double stranded "activated" DNA. Since EtBr binds almost exclusively to double stranded DNA under the conditions of the Mullis PCR, and would thus be present at an even higher ratio of EtBr to double stranded (i.e., inhibitable) DNA, there is even less scientific basis to lead one of skill to rely on the teachings of Kaledin to suggest that low levels of EtBr would not inhibit PCR.

Finally, if the Examiner maintains that the first and second reasons are not persuasive, a closer examination of the mechanism of the multi-step PCR of Mullis reveals that each extension must be complete in order to have exponential amplification and each newly synthesized duplex must be fully denatured in every cycle, in contrast to the prior art's single step polymerase activity assays. The mechanism by which EtBr inhibits primer extension and inhibits DNA denaturation makes the presence of EtBr much more of a concern in PCR. This is further evidence that there is no scientific basis to lead one of skill to rely on the teachings of Kaledin to suggest that low levels of EtBr would not inhibit PCR.

#### 7. THE PHYSICOCHEMICAL MECHANISM FOR INHIBITION OF DNA SYNTHESIS BY ETBR.

With regard to my basis for stating that Kaledin *et al.* fails to motivate the use of low amounts of EtBr in a PCR, an understanding of the biophysical interaction of EtBr with nucleic acid and its mechanism for inhibiting DNA polymerization may help to clarify Applicant's position. EtBr is a noncovalent nucleic acid binding agent. It binds by inserting itself (*i.e.*, intercalating) between the base pairs of the double helix. In this manner it often acts as an inhibitor of DNA or RNA synthesis. As intercalating agents become inserted into a DNA molecule, they increase the spacing of successive base pairs along the helical axis to roughly 7Å, almost the distance between phosphate atoms in a fully extended chain. In this situation, very little rotation is possible around the helical axis, resulting in not only an extension of the double helix, but extensive unwinding of the helix as well. Thus, when a molecule of EtBr intercalates, the rotation angle between the two adjacent base pairs is reduced from 36° to 10°. In covalently closed, circular DNA molecules, intercalation of EtBr (and thus unwinding) results in the introduction of compensatory superhelical turns. As more and more of the EtBr molecules intercalate, the DNA molecule will become more twisted, until it is unable to twist any more, at which point no more EtBr molecules can be bound. On the contrary, a linear DNA molecule or a nicked circle does not have the topological constraint of reverse twisting, and can continue to bind more EtBr molecules until saturation is reached.

(approximately 1 EtBr molecule for every 2 base pairs). EtBr also binds in a non-intercalating fashion to single stranded DNA and RNA in low salt ( $\leq 10$  mM), albeit with a much lower affinity.

With the above understanding of the binding interactions between EtBr and DNA, we can now address the interaction between the EtBr and the polymerase enzyme. In short, there is no significant interaction. The EtBr binds to the DNA template and not to the enzyme. The intercalator is not a polymerase inhibitor. More specifically, EtBr does not interact directly with the DNA polymerase molecule, nor does it exert its influence on nucleic acid synthesis via inhibition of the polymerase molecule. Thus, although Kaledin *et al.* (*Biokhimiya* 1981, 46:1576-1584) refer to EtBr as a DNA polymerase activity inhibitor, they correctly identify its mode of action as an "intercalating template poison". Therefore, the  $ID_{50}$  and  $ID_{min}$  concentrations reported by Kaledin *et al.* in Table 3, reflect not the concentrations of EtBr which adversely affect the DNA polymerase, but those concentrations which "poison" the DNA template substrate, and thereby inhibit DNA synthesis. This "poisoning" can occur in several manners. Intercalation of the EtBr into the duplex primer-template region will alter the conformation of the helix as described above, thus preventing efficient binding and extension of the 3' end of the primer by the polymerase molecule. Additionally, if the substrate is primarily nicked and gapped duplex DNA (*i.e.*, "activated" DNA), intercalation by EtBr into double stranded regions 3' of the primer will distort the helix, inhibiting downstream nick-translation synthesis. And finally, if the substrate is primarily single stranded, as in PCR, EtBr will intercalate into downstream double stranded hairpin loop secondary structures, stabilizing these structures as described below, and thus inhibiting downstream polymerization (*i.e.*, strand-displacement synthesis).

In other words,  $ID_{50}$  and  $ID_{min}$  EtBr concentrations are not properties of the enzyme *per se*, but are properties of the amount and conformation of the DNA template in the reaction. As described above, the amount of EtBr that binds to DNA is dependent on the DNA conformation (covalently closed, circular double stranded DNA vs. nicked circle or linear double stranded DNA vs. single stranded DNA). What Kaledin *et al.* teaches then is that amount of EtBr which inhibits DNA synthesis on 8  $\mu$ g of "activated" calf thymus DNA per 50  $\mu$ l reaction.

8. KALEDIN TEACHES AWAY FROM THE INVENTION BECAUSE THE RATIOS OF ETBR TO DNA IN KALEDIN WOULD LEAD ONE OF SKILL TO PREDICT THAT SIGNIFICANT INHIBITION WOULD BE PRESENT IN A PCR REACTION USING THE CONCENTRATIONS OF ETBR RECITED IN THE SPECIFICATION.

In the simplest case of providing an analytic rebuttal of the *prima facie* case of obviousness, if one calculates the ratio of EtBr to DNA reported by Kaledin *et al.* as 50% inhibitory, it is surprisingly discovered that this ratio is not inhibitory in the context of a PCR amplification. In brief, I have calculated that the ratios of EtBr to DNA in the PCR mixtures of the invention are typically within the range in which one of skill would expect substantial inhibition based on the teachings of Kaledin. Surprisingly, there are no significant inhibitory effects from the EtBr.

The ratios are calculated as the ratio of EtBr molecules to total amount of DNA per reaction mixture initially present in the reaction. The Kaledin reference teaches that a ratio of  $1.4 \times 10^{-4}$   $\mu\text{mol EtBr} : 1 \mu\text{g DNA}/50 \mu\text{l Rx}$  = 50% inhibition and a ratio of  $3.1 \times 10^{-5}$   $\mu\text{mol EtBr} : 1 \mu\text{g DNA}/50 \mu\text{l Rx}$  = minimal inhibition. Although the Examiner may have originally understood Kaledin to teach that the polymerase is poisoned by concentrations of EtBr greater than 5  $\mu\text{M}$ , the correct interpretation is that greater than  $3.1 \times 10^{-5}$   $\mu\text{mol EtBr}$  is needed to bind to double stranded regions of 1  $\mu\text{g}$  of Kaledin's activated DNA before it binds to a significant portion of the template in those regions as described above to impair 3' primer extension.

The surprising results of the Applicant's work is apparent when you normalize their EtBr to DNA ratios of the examples on pages 22-29 of the specification. The ratio of  $\mu\text{mol}$  of EtBr to 1  $\mu\text{g}$  of DNA per reaction ranges from  $1.2 \times 10^{-3}$  to  $1.2 \times 10^{-4}$   $\mu\text{mol EtBr}$ . These ranges are well above the ratio of EtBr to DNA ( $3.1 \times 10^{-5} : 1$ ) reported by Kaledin to exhibit minimal inhibition of DNA synthesis.

Accordingly, those of skill reading Kaledin would have concluded that the prior art's use of EtBr would have had a grossly negative effect on the amplification of target nucleic acid in a typical PCR where DNA is typically present in significantly lower concentration than used by Kaledin. They would have intuitively understood that the ratios of binding sites of the target DNA to EtBr would be so high in a typical PCR mixture when EtBr was present even at 5  $\mu\text{M}$  that the PCR amplification would have been significantly hampered. And even if the EtBr:DNA ratio of the Applicant's invention was reduced to be in line with the teaching of Kaledin to provide for "minimal inhibition" of amplification, the concentrations of EtBr would be so low in a typical PCR mixture that the detection of the signal would not be practical because of the problems of sensitivity.

The data in the Applicant's specification and in the reference were normalized by converting it to micromoles EtBr : micrograms DNA per reaction. The ratio thus reflects the amount of DNA present in the mix and the number of EtBr molecules (available for binding).

In the subject application there are six examples. The following conversions are appropriate:

Example I

0.51  $\mu$ M EtBr : 0.407  $\mu$ g DNA/100  $\mu$ l Rx =

0.00005 micromoles EtBr : 0.407  $\mu$ g DNA/100  $\mu$ l Rx =  $1.2 \times 10^{-4}$

Example II

1.27  $\mu$ M EtBr : 0.109  $\mu$ g DNA/100 $\mu$ l Rx =

0.000127 micromoles EtBr : 0.109  $\mu$ g DNA/100  $\mu$ l Rx =  $1.2 \times 10^{-3}$

Example III

0.000127 micromoles EtBr : 0.22 - 0.28  $\mu$ g DNA/100  $\mu$ l Rx =  $5.8 \times 10^{-4}$  -  $4.5 \times 10^{-4}$

Example IV

Not applicable

Example V

0.000127 micromoles EtBr : 0.159  $\mu$ g DNA/100  $\mu$ l Rx =  $8.0 \times 10^{-4}$

Examples VI

1.27  $\mu$ M EtBr : 0.817  $\mu$ g DNA/50  $\mu$ l Rx =

0.000635 micromoles EtBr : 0.817  $\mu$ g DNA/50  $\mu$ l Rx =  $7.8 \times 10^{-4}$

The Kaledin *et al.* reference discloses:

160  $\mu$ g DNA/ml = 8  $\mu$ g/50  $\mu$ l reaction

23  $\mu$ M EtBr : 8  $\mu$ g DNA/ 50  $\mu$ l Rx =

0.00115 micromoles EtBr : 8  $\mu$ g DNA/50  $\mu$ l Rx =  $1.4 \times 10^{-4}$  = 50% inhibition

5  $\mu$ M EtBr : 8  $\mu$ g DNA/50  $\mu$ l Rx =

0.00025 micromoles EtBr : 8  $\mu$ g DNA/50  $\mu$ l Rx =  $3.1 \times 10^{-5}$  = minimal inhibition

In view of the above discussion of EtBr:DNA ratios comparing the Kaledin ratios versus the ratios disclosed by the Applicant, it is submitted that one of skill reading Mullis, Sutherland, and Kaledin would not be motivated to combine low amounts EtBr in a PCR mixture for purposes of monitoring the rate of amplification during a PCR, and would be surprised to discover that the EtBr at the ratios disclosed by Kaledin would not inhibit the PCR. The ratios reported by Kaledin as inhibiting their polymerase assays are the same ratios used by the Applicant in their examples.

9. THE INHIBITORY PROPERTIES OF THE ETBR CONCENTRATIONS REPORTED BY KALEDIN ARE NOT APPLICABLE TO THE MULLIS PCR BECAUSE THE STARTING DNA OF THE REACTION MIXTURES OF KALEDIN ARE DOUBLE STRANDED WHILE THE STARTING DNA OF MULLIS IS SINGLE STRANDED.

It is my understanding that the Examiner wishes to combine the teachings of Kaledin with the Mullis patent. The Examiner concludes that the 5  $\mu$ M levels of EtBr used by Kaledin in combination with the teachings of Sutherland would motivate the addition of EtBr to Mullis' PCR. In this section of my declaration, I will explain that the reaction mixtures of Kaledin and Mullis are very different. The Kaledin mixtures primarily contained double stranded DNA which binds EtBr strongly, while the Mullis reactions primarily contain single stranded DNA (in the form of the primers).

Because of the significant binding differences between EtBr and single or double stranded DNA, it would not be possible for one of skill to infer from Kaledin that a PCR reaction could be performed in the presence of useful levels of EtBr without any significant inhibitory effects. The overall conformation of the DNA in a Mullis PCR, in conjunction with the a more sophisticated understanding of the mechanism of EtBr binding and inhibition, leads to the conclusion that there is no reasonable scientific basis for one of skill to compare the nucleic acid in a PCR with the DNA templates used by Kaledin *et al.* Thus, it would also follow that one of skill would not conclude with any reasonable degree of certainty that low levels of EtBr reported by Kaledin will effectively permit PCR to be monitored during amplification without significant inhibition.

More specifically, the activated DNA of Kaledin consists primarily of linear double stranded DNA containing nicks and small gaps. Conversely, the DNA in a PCR reaction is primarily excess unannealed single stranded primers and single stranded template with a short stretch of annealed primer-template duplex during the anneal/extension phase of the reaction, since this directly follows a denaturation phase in every cycle. As noted above, both the affinity and the

inhibitory effect of EtBr are negligible for single stranded DNA, especially in the "high" salt conditions (*i.e.*, 50 mM KCl and 1.5-5 mM MgCl<sub>2</sub>) present during PCR. This is because on double stranded DNA there are a large number of high-affinity binding sites for the molecule, only some percentage of which will interfere with DNA synthesis. In contrast, in a primarily single stranded DNA reaction, such as PCR, the only high-affinity binding sites for EtBr are the duplex region of the primer-template where the polymerase needs to bind and extend, and the downstream regions of secondary structure where the polymerase needs to "read through" in order to get the required complete replication of the template strand. Thus, the concentration of EtBr needed to inhibit synthesis on primarily double stranded DNA will be much different from that which inhibits oligomer-primed single stranded DNA. One of skill would have no basis to conclude, as the Examiner has, that the low levels of EtBr taught by Kaledin might avoid inhibition of PCR while permitting one to monitor amplification.

Thus, as one gives more considered thought to the problem, the Kaledin teaching would again teach away from the use of EtBr in a PCR. The small amount of double stranded DNA in a PCR would be expected to exacerbate the effect of EtBr. As the Examiner may already recognize, the amount of DNA at any stage of a PCR is also considerably less than that in an activity assay as performed by Kaledin *et al.*, even though the amount of DNA is increasing exponentially with each cycle. The starting amounts of DNA vary, but are typically in the range of 100-800 ng (input DNA + primer) per 50 µl reaction (see examples in the present specification). PCR amplifications rarely produce product in excess of a few µg per 50 µl reaction at the end of the thermal cycling.

Because there is very little double stranded DNA in the PCR reaction to "bind up" the EtBr in solution with high affinity, the amount of free ethidium in the PCR is presumably higher at the total concentrations that Kaledin suggests to be minimally inhibiting in their polymerase activity assay. Thus, one of skill would expected EtBr to be even more detrimental to the small amount of double stranded DNA (*i.e.*, primer-template duplex and hairpin loops) present in the invention.

10. THE PHYSICOCHEMICAL MECHANISM FOR INHIBITION OF POLYMERASE ACTIVITY PREDICTS THAT THE ASSAYS OF KALIEDIN AND SUTHERLAND WOULD BE SIGNIFICANTLY LESS SENSITIVE TO ETBR INHIBITION THAN PCR.

Finally, as stated above, PCR is a multi-step reaction that is mechanistically significantly more complex from a physicochemical perspective than the single-step polymerase activity assays of either Kaledin or Sutherland. More specifically, the need for complete full-length replication of the template strand and repetitive denaturing of duplexes are not a part of the Kaledin or Sutherland assays. Both full extension and complete denaturation are integral to a successful PCR and both processes are expected to be inhibited by the presence of EtBr. For this reason, once again, one of skill could not predict with a reasonable expectation of success that the addition of EtBr, even at the low concentration (5  $\mu$ M) suggested by Kaledin, would be free of negative effects upon PCR.

It is useful to consider the physicochemical aspects of both the polymerase activity assays and PCR. The mechanism for inhibition of polymerase activity depends upon the ability of the EtBr to intercalate into the double stranded regions of the template nucleic acid and thereby inhibit polymerase binding and/or 3' extension of the primer, in the ways discussed above. It is again important to reinforce that the polymerase molecule itself is not poisoned by the EtBr. The correct mechanism views the polymerase as both unable to bind to and extend the distorted EtBr-containing 3' primer-template region as well as unable to continue to synthesize through regions of EtBr-containing downstream double stranded DNA via the process of nick translation or strand displacement synthesis. It is important to note that the intercalation of EtBr into double stranded DNA considerably stabilizes the duplex structure, as well as distorting the helix, thus making it additionally difficult to read through such structures in order to get the requisite full-length extension of the template strand. Instead, the enzyme simply stops its extension and is released from the template-primer complex, free to find another template/primer combination. Because the polymerase assays of Kaledin and Sutherland rely only on the incorporation of dNTP into a single cycle of extension, and not the completed synthesis of the entire template strand, the assays are relatively insensitive to inhibition by EtBr.

In contrast, PCR requires that the polymerase dependent 3' extension process go to completion. It is not sufficient for the polymerase to partially extend the primers, and finding further extension blocked by EtBr-stabilized and -distorted duplex hairpin structures, to release and to find another template/primer combination. For without full extension, the primer extension products of the previous cycle cannot function as a template in the next cycle. For this reason,

PCR is going to be exquisitely sensitive to any compound that inhibits full extension unlike the assays of the Kaledin and Sutherland references.

In addition, and equally important as the above points, is the fact that the polymerase activity assays of the prior art do not require denaturation of the double stranded DNA that they are detecting. PCR requires denaturation of the duplexes formed in the previous extension phase in order to provide the exponential amplification that is the hallmark of the reaction. EtBr dramatically increases DNA duplex stability. For over twenty-five years it has been well known that EtBr intercalation can increase the denaturation temperature ( $T_m$ ) by about  $10^\circ - 20^\circ \text{C}$ . Thus, if one adds a helix stabilizing agent to the reaction, the  $T_m$  of the duplexes will increase dramatically, and the kinetics of the polymerase chain reaction would be expected to change. It has already been explained by Applicant that the inhibition of PCR, by even a few percent, dramatically impacts on the final amount of product.

A recent review article to be published this year expressly states this fact. The review article by Wittwer *et al.*, is entitled *Rapid Cycle DNA Amplification*. It is to be published as Chapter 15 of the Polymerase Chain Reaction, Edited by Mullis, Ferre, and Gibbs. A copy is attached as Exhibit 2. At the carryover paragraph on pages 175-176 of the preprint the authors state:

Ethidium bromide can also be included at concentration used for staining gels ( $0.5 \mu\text{g/ml}$ ) without apparent changes in yield or specificity (Fig. 15.2C). This is surprising because of the known influence of ethidium bromide on DNA melting (Meada *et al.*, 1990). The potential for using ethidium bromide fluorescence during amplification as a monitor of double stranded DNA production is attractive.


I agree with the above statement of Wittwer *et al.* The addition of EtBr prior to conducting PCR is contrary to conventional wisdom. The PCR amplifications are more complex than the polymerase activity assays of Kaledin and Sutherland. Unlike the prior art polymerase activity assays of Kaledin and Sutherland, the PCR 3' extensions must go to completion and be repetitively denatured to function properly. With the above explanation of the mechanism of EtBr inhibition of polymerase reactions, it now should be exceptionally clear that one of skill in PCR would not view Kaledin as motivating the use of low levels of EtBr in the PCR reaction mixtures of Mullis with any scientific rational to support a reasonable expectation of a successful result.

Conclusion

Herein I explain that the physicochemical attributes of PCR are so different from the polymerase activity assays of Kaledin that comparisons with regard to EtBr inhibition are not possible. I further explained that the binding characteristics of EtBr to the single stranded DNA of PCR assays are so different from the binding characteristics of EtBr to the double stranded DNA of Kaledin that the minimal inhibitory effects of EtBr reported by Kaledin at 5  $\mu$ M would not be meaningful in the context of a PCR. In addition, it was explained that the EtBr to DNA ratios reported by Kaledin as inhibitory are surprisingly not inhibitory to a PCR.

This Declarant has nothing further to say.

Date: January 20<sup>th</sup>, 1994

  
Richard D. Abramson